Herein is described a good medium for growing pertussis cultures for vaccine; one easy to prepare and inexpensive.

Beef-Heart Charcoal Agar for the Preparation of Pertussis Vaccines

LUCY MISHULOW, LESLIE S. SHARPE, AND LILLIAN L. COHEN

City Department of Health, Bureau of Laboratories, New York, N. Y.

THE utilization of charcoal as a substitute for blood or serum was reported by Glass and Kennett (1939) [1] who found that *Neisseria gonorrheae* and *Neisseria intracellularis* grew freely on nutrient agar containing charcoal. Pollock (1947) [2] obtained a good growth of a strain of *Hemophilus pertussis* on charcoal agar, but this strain also grew freely on 10 per cent blood agar and on blood-free starch agar and was apparently not in phase I. More recently, Powell, Culbertson, and Esminger (1951) [3] reported that they had obtained good growth of *H. pertussis* phase I on charcoal agar which was incorporated in the Cohen-Wheeler [4] synthetic broth, and that pertussis vaccines prepared on this medium were, in most instances, as potent as the National Institutes of Health reference vaccine.

We were able to obtain some of the charcoal agar through the courtesy of Dr. Powell who also sent us a generous supply of the charcoal which was used in their medium. We prepared charcoal agar according to the Powell, *et al.*, formula and tested this together with the charcoal agar sent to us by Dr. Powell. Cultures of *H. pertussis* phase I grew equally well on both lots of the charcoal agar. The growth was smooth, the organisms were morphologically typical, and agglutinated well with anti-pertussis phase I serum.

The incorporation of charcoal agar in synthetic broth seemed to have a distinct advantage over synthetic broth as such. In our experience the growth of *H. pertussis* phase I in the different preparations of synthetic broth [4-9] was not uniformly satisfactory. Some of the strains of *H. pertussis* phase I failed to grow in the liquid media, and the concentration of organisms of the strains that did grow frequently varied within wide limits—not only in the different preparations of the synthetic broth but also in different bottles of the same preparations. We, therefore, continued to manufacture our pertussis vaccines on the Bordet-Gengou medium which has given uniformly good results over a long period of years. The medium of Powell, Culbertson, and Esminger gave such promising results that it seemed desirable to investigate it further as a substitute for the Bordet-Gengou medium. It occurred to us, however, that a simpler base than the synthetic broth used in the Powell, *et al.*, medium might give equally good results. With this object in view we carried out the following studies.

We used in our preliminary tests glycerine-potato extract agar, which is the base of the Bordet-Gengou medium, and beef-heart infusion agar to each of which 0.4 per cent of charcoal was added. These two media were tested with a number of strains of *H. pertussis* phase I.

The growth on the glycerine-potato charcoal agar was unsatisfactory. A number of the strains failed to grow, and those that did grow on the first generation failed to grow on subsequent transplants. The growth on the beef-heart charcoal agar was more promising. Although some of the strains failed to grow on this medium, also, a fairly good growth was obtained with some of the other strains. We continued our tests of the beef-heart charcoal agar using additional enrichments such as soluble starch, yeast extract, arabinose, and casamino acid. These reagents were used singly, as well as in various combinations.

After considerable experimentation we found that beef-heart charcoal agar that contained 1 per cent soluble starch and 0.35 per cent yeast extract gave excellent growth of *H. pertussis* phase I. This medium was prepared as follows:

Beef-Heart Charcoal Agar

Bacto-peptone (Difco)	10	gm.
Sodium chloride	5	"
Soluble starch (Merck)	10	"
Yeast extract (Difco)	3½	"
Agar agar	35	"
Beef-heart infusion	1,000	ml.*

* 500 grams beef-heart in 1,000 ml. H_2O

The reagents were dissolved over an open flame and the reaction adjusted to pH 7.4–7.6. Then, 4.0 grams of charcoal (Norit SG) were added, mixed well, and distributed into tubes or bottles. It was sterilized at 15 pounds pressure for one-half to one hour according to the size of the containers. The reaction after sterilization was usually pH 7.2–7.4.

This medium was tested with a number of strains of *H. pertussis* phase I. All but three of the strains tested grew more abundantly on the charcoal agar than on the control Bordet-Gengou medium. The three strains that did not grow abundantly on the charcoal agar also grew delicately on the Bordet-Gengou medium.

Some of the strains of *H. pertussis* were carried through 11 generations on the charcoal medium by transplanting them at 48-hour intervals. At each transplant the growth was examined by smear and was tested for agglutination with antipertussis phase I serum. The organisms remained morphologically typical and stained well by the Gram method throughout this experiment. They agglutinated to titer with antipertussis phase I serum through the ninth generation. After that some of the strains agglutinated poorly, thus indicating a change in phase.

The results obtained in these tests showed that *H. pertussis* phase I grew as freely and abundantly on the beef-heart charcoal agar as on the synthetic-broth charcoal agar of Powell, *et al.*

Our next problem was to determine whether potent pertussis vaccines could be prepared on this medium.

PREPARATION OF PERTUSSIS VACCINES ON BEEF-HEART CHARCOAL AGAR

Although no change of phase could be detected during a number of transplants of *H. pertussis* phase I on the beef-heart charcoal agar, it seemed advisable to carry the vaccine strains on 30 per cent sheep-blood Bordet-Gengou medium on which, in our experience, *H. pertussis* can be maintained in phase I for many months. Preliminary cultures for the vaccine were made by inoculating beef-heart charcoal agar in 1-inch tubes with the 48-hour growth on Bordet-Gengou medium. These were incubated at 35° C. for 48 hours. The growth on the tubes was suspended in 10–12 ml. of 1 per cent casamino acid of pH 7.2–7.4, and about 4 ml. of the suspension was inoculated on the charcoal agar in 1-liter bottles. The bottles were incubated for 24 hours at 35° C. They were then tilted back and forth to redistribute the growth over the entire surface of the

medium and were reincubated for another 24 hours. The 48-hour growth on the bottles was suspended in sterile physiological saline which contained 1–5,000 merthiolate. The growth was profuse, smooth, nonadherent and emulsified readily. Before pooling the growth, each bottle was examined by smear for purity and typical morphology. The saline suspension was then distributed aseptically into sterile bottles. Each of the bottles of the pooled growth was again tested for purity by inoculating a small amount of the vaccine on tubes of Bordet-Gengou medium, on blood-free agar, and into glucose broth in fermentation tubes in order to detect aerobic and anaerobic contaminants. These tubes were incubated at 35° C. for seven days. Growth of *H. pertussis* usually appeared within two to three days on the Bordet-Gengou medium and was typical culturally and morphologically. There was no growth on the blood-free media even after seven days incubation unless there was contamination. Bottles of the contaminated vaccine were discarded.

As soon as the tests for purity were made, the vaccine was incubated at 35° C. overnight in order to hasten the sterilizing effect of the merthiolate, and was then stored at 8° to 10° C.

The vaccines prepared on the charcoal agar usually contained a small amount of charcoal which was washed off with the bacteria from the medium. The charcoal was readily removed by filtration through sterile 40-mesh gauge of monel metal to remove clumps and through 200-mesh sterile nylon. Should a trace of charcoal remain after filtration, it can be removed by allowing the vaccine to sediment for several days in the ice chest and siphoning off the vaccine without disturbing the sediment. Using this method, only a negligible amount of the vaccine is lost through filtration and sedimentation, as the bacteria do not readily settle out on short storage.

After the purity tests were completed and the vaccines were filtered to remove the charcoal, they were standardized by means of a photoelectric cell to determine the concentration of the bacteria per ml. and were tested for sterility in the same manner as described for purity. In most instances the vaccines were sterile after several weeks storage.

A number of pertussis vaccines have been prepared on the beef-heart charcoal agar in the past four years. In each instance the growth was profuse and smooth, the organisms were morphologically typical, and the saline suspensions were homogeneous. Thirty-seven of the vaccines were tested for potency.

PRELIMINARY TESTS FOR POTENCY OF PERTUSSIS VACCINES PREPARED ON BEEF-HEART CHARCOAL AGAR

In testing the vaccines for potency it was expedient to combine some of the preparations in order to economize on mice, since large numbers of mice are required in each test. In the preliminary tests, three single vaccines and six combined preparations were used which represented a total of 16 vaccines. The tests were carried out according to the requirements of the National Institutes of Health. Several dilutions of each vaccine and of the National Institutes of Health reference vaccine were injected intraperitoneally into groups of susceptible mice of standard weights. A group of unvaccinated mice of the same lot and the same weights were set aside to serve as controls. Ten to 14 days after vaccination the immunized mice were challenged intracerebrally with 100,000 bacilli of a virulent culture of *H. pertussis* suspended in 1 per cent casamino acid solution of pH 7.2–7.4. The unvaccinated controls were divided into groups and injected intracerebrally with the challenge dose, and with smaller doses, in order to determine the virulence of the culture on the day of the test. Several of the small doses were

TABLE 1

Potency of Pertussis Vaccines Prepared on Beef-Heart Charcoal Agar

	Preliminary Tests		
Vaccine	*ED 50 × One Million*	*Reference Vaccine ED 50 × One Million*	*Remarks*
P149A	135		
P150	175	1,280	
P151	185		Vaccines P149A P150, and P151 are single vaccines, M25-M33 are combinations of 2-3 vaccines.
M25	450	1,160	
M26	1,070		
M24	375	1,000	
M31	670		
M32	400	1,600	
M33	240		

cultured on plates of Bordet-Gengou medium to determine the number of viable organisms in the given doses. The smallest number of the bacilli that killed 50 per cent of the control mice (LD_{50}) were used as the basis for calculating the virulence of the culture.

The vaccinated and control mice were observed for 14 days and the results are given in Table 1. No attempt was made in the preliminary tests to evaluate the results statistically. The ED_{50}, or the smallest amount of vaccine that protected 50 per cent of the mice for the period of observation, was estimated according to the Reed-Muench Method.[10]

The results obtained in the potency tests showed that eight of the nine preparations on the charcoal agar were considerably more potent than the reference vaccine, and the ninth was approximately as potent as the reference vaccine.

Tests for potency were then carried out on seven additional preparations of pertussis vaccines that were made on the charcoal agar and on six of the preparations that were tested in the preliminary studies (Table 1). These 13 preparations represent 34 individual vaccines. The results of these tests were evaluated statistically by the Wilson-Worcester [11] method and are charted in Table 2.

The results tabulated in Table 2 show that 12 of the 13 preparations tested were more potent than the reference vaccine and the thirteenth (M26) was approximately as potent as the reference vaccine. This last preparation is the same as that tested in the preliminary studies with the same result (Table 1 M26).

DISCUSSION

It was found that beef-heart agar containing 0.4 per cent charcoal, 1 per cent soluble starch, and 0.35 per cent yeast extract gave a very good growth of *H. pertussis* phase I. The organisms grew rapidly and profusely and the growth was very smooth, emulsified readily, and made a homogeneous suspension. The organisms were morphologically typical, stained well by the Gram method, and remained in phase I for a number of generations. The beef-heart charcoal agar proved to be an excellent medium for the preparation of pertussis vaccines. During the past four years a large number of pertussis vaccines were prepared on this medium and they were uniformly of high concentration. Thirty-seven of the vaccines were tested for potency; four of these were tested individually and 33 were combined into 12 preparations. All of these preparations, except one, were more potent than the reference vaccine, and that one was approximately as potent as the reference vaccine.

TABLE 2

Potency of Pertussis Vaccines Prepared on Beef-Heart Charcoal Agar

Vaccine		ED_{50} in Millions	Log ED_{50} ± 1 S.D.	Statistical Evaluation: ED_{50} Limits 1 S.D. in Per cent	Statistical Evaluation: ∝ ± 1 S.D.	∝ 2 S.D. Limits	Challenge Culture $I.D_{50}$: Bacilli	Challenge Culture $I.D_{50}$: Limits 1 S.D. Per cent
Charcoal	M22	910	1.04 ± 0.16	69–143	1.14 ± 0.34			
"	M26	1,570	0.81 ± 0.34	221–450	0.54 ± 0.29		125	0
Ref. Lot	4/51	1,190	0.92 ± 0.11	77–130	1.92 ± 0.53	2.98–0.86		
Charcoal	M24	490	1.40 ± 0.16	77–129	1.94 ± 0.52			
"	M31	700	1.15 ± 0.19	65–155	1.00 ± 0.33		20	0
Ref. Lot	4/51	1,300	0.88 ± 0.17	68–147	1.11 ± 0.36	1.83–0.39		
Charcoal	M23	390	1.71 ± 0.13	75–134	1.47 ± 0.40		150	90–111
Ref. Lot	4/52	1,700	1.05 ± 0.31	49–206	0.62 ± 0.29	1.19–0.04		
Charcoal	M35	145	1.83 ± 0.22	60–165	0.96 ± 0.38			
"	P163	550	1.25 ± 0.12	75–133	1.57 ± 0.43		130	69–143
Ref. Lot	4/52	1,160	0.94 ± 0.11	77–129	1.87 ± 0.51	2.89–0.75		
Charcoal	M25	402	1.70 ± 0.18	66–151	0.93 ± 0.31		130	58–173
Ref. Lot	4/53	802	1.40 ± 0.09	81–123	2.59 ± 0.75	4.10–1.08		
Charcoal	M32	490	1.31 ± 0.17	68–147	1.00 ± 0.32		158	74–136
"	M33	145	1.83 ± 0.22	60–165	0.96 ± 0.38			
Ref. Lot	4/53	1,780	0.75 ± 0.11	77–129	1.98 ± 0.58	3.06–0.90		
Charcoal	M34	158	1.80 ± 0.18	66–151	0.97 ± 0.32			
"	M36	148	1.83 ± 0.16	70–144	1.16 ± 0.35		220	63–158
"	M37	148	1.83 ± 0.16	70–144	1.16 ± 0.35			
Ref. Lot	4/53	316	1.80 ± 0.24	57–174	0.70 ± 0.29	1.28–0.12		

The results that we obtained on the beef-heart charcoal agar correspond to those that were obtained by Powell, Culbertson, and Esminger on the synthetic-broth charcoal agar. The beef-heart charcoal agar, however, has the advantage of being a simpler medium than the synthetic-broth charcoal medium, since it requires fewer ingredients and, consequently, it is easier to prepare and is less expensive.

The beef-heart charcoal agar has several advantages over the Bordet-Gengou medium: (1) it eliminates the labor and expense of obtaining sterile blood which is required in the latter; (2) it reduces the loss of vaccine through contaminations which frequently occur when vaccines are prepared on a large scale on the Bordet-Gengou medium, because the blood must be added to this medium after sterilization, whereas the charcoal agar is completely sterilized before it is used; and (3) more concentrated vaccines are obtained on the charcoal agar than on the Bordet-Gengou medium, because the organisms grow more profusely on the charcoal agar than on the Bordet-Gengou medium.

Although our investigation showed that strains of *H. pertussis,* cultured on the beef-heart charcoal agar, remained in phase I for nine generations, it seemed advisable to maintain the cultures that were used in vaccine preparation on Bordet-Gengou medium in order to insure against any possibility of deterioration during the preparation for the vaccine. In our experience, cultures of *H. pertussis* phase I showed no change of phase after cultivation on the Bordet-Gengou medium for a number of months. By using the method of inoculating the preliminary cultures for the vaccines from the Bordet-Gengou medium to beef-heart charcoal agar, which in turn were inoculated on the charcoal agar in bottles, highly satisfactory results were obtained.

SUMMARY AND CONCLUSIONS

Beef-heart charcoal agar has been found to give a rapid and profuse growth of *Hemophilus pertussis* phase I. The growth was smooth, the organisms were typical morphologically, culturally, and serologically.

A number of pertussis vaccines were prepared on this medium, and the growth was uniformly highly satisfactory. It was smooth, nonadherent, emulsified well, and showed no tendency to clump on storage. To date, four individual vaccines and 12 combined preparations have been tested for potency. These 12 preparations represent 33 individual vaccines, giving a total of 37 vaccines tested. All except one of the vaccines were higher in potency than the National Institutes reference vaccines, and the one exception was approximately equal to that of the reference vaccine.

Although a comparatively small number of the vaccines prepared on the beef-heart charcoal agar were tested for potency, the results obtained in this study indicate that this medium is excellent for the preparation of pertussis vaccines. The medium is easy to prepare and is inexpensive, and for these reasons it promises to be the medium of choice for the preparation of pertussis vaccines.

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Annually More School Children

"The remaining years of the present decade will witness a steady rise in our school age population, their number increasing by an average of more than 1,000,000 annually." Thus the August, 1953, *Statistical Bulletin* of the Metropolitan Life Insurance Company introduces its discussion of what the country may expect in school age population just ahead. By 1960 it estimates there will be a growth of more than one-fourth in number of children 5–17 years of age. Without any allowance for interstate migration, the increase between 1953 and 1960 will be more than a third in the West and more than one-fifth in the South. The percentage increase will be greater among those 14–17 years than among the younger group. The estimates for the older age group have been carried to 1964 by which time an increase of more than 60 per cent will have taken place and of more than three-fourths in the West.

The article concludes, "The problems of providing adequate school facilities and teaching staffs to meet the needs of the rapid growth of our school age population are already with us. These problems will be even larger in the years ahead, particularly at the secondary school level."